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**Remarks**

Claims 1, 3-6 and 8 to 24 are currently pending in this application, of which only claims 1, 11, 23 and 24 are independent claims. Amendments to the claims were introduced to further clarify the claim language.

The specification has been amended to insert a claim to priority under 37 CFR §1.78 using the procedure specified in the Official Gazette of October 2, 2001. Applicant notes that the priority claim was recognized by the Office in the filing receipt dated March 21, 2002.

***Memorandum of Interview***

On June 22, 2004 applicant's representative was granted a personal interview with Examiner Afremova. During this interview all pending claims were discussed as well as the prior art, in particular the Bianchi and Saunders references cited in the Office Action of December 30, 2004. A claim format such as currently claimed in claim 11 was discussed as well as the importance of low pH values in the tissue culture mixture of the presently claimed invention. Applicant was advised to provide a declaration to outline the importance of low pH.

***Claim Objections***

On page 2 of the Action, the Office objects to the reference to Figure 1 in claim 9 and suggests using claim language identical to the claim language used in U.S. Patent No. 6,309,606.

In response, applicant has amended claim 9 to incorporate the limitations of claim 1 of said patent.

***Rejections under 35 U.S.C. §112, second paragraph***

Also on page 2, the Office rejected claims 1, 3-9 under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the

subject matter which applicant regards as the invention.

In particular, the view was expressed that claims 1 and 7 are indefinite because it is uncertain what “appropriate” identification procedures are.

In response, applicant has amended claims 1 solely to clarify the claim language. New claim 24 contains corresponding language. Support for this language can be found on page 6, lines 23 to 24.

On pages 2 and 3, the view was expressed that claims 1 and 7 were indefinite with regard to the components of the “tissue culture medium” because it was uncertain what components of this medium would modify the mixture comprising “tissue culture medium”, “maternal blood” and “aqueous solution” so that the final mixture would have the claimed characteristics. In particular, the view was expressed that, e.g., the source of glucose in the mixture was uncertain, since neither the “tissue culture medium” nor the “aqueous solution” clearly indicated the inclusion of glucose. Thus, it was argued, that the claims were incomplete for omitting essential elements. Reference was made to MPEP §2172.01.

A claim can be rejected as incomplete if it omits essential elements, steps or necessary structural cooperative relationships of elements, such omission amounting to a gap between the elements, steps or necessary structural connections. See <http://www.uspto.gov/web/offices/pac/dapp/35usc112.htm> (Deputy Commissioner for Patent Examination Policy on MPEP §2172, citing In re Collier, 397 F.2d 1003, 158 USPQ 266 (CCPA 1968).

However, greater latitude is permissible with respect to the definition in a claim of matters not essential to novelty or operability than with respect to matters essential thereto. Furthermore, the breadth of a claim is not to be equated with indefiniteness. See MPEP 2173.04.

Applicant respectfully submits that no essential element was omitted from the claim (see page 5, lines 10 to 13, page 5, line 32 to page 6, line 4). Also there is no requirement that applicant specifies the source of an element of a claim. Claim 1 as

amended clearly recite the elements of the tissue culture mixture and requires that this tissue culture mixture comprises tissue culture and maternal blood. However, the claim is an open ended one, thus allowing for the addition of components in each of the recited steps.

However, Applicant also includes new independent claim 24, which, apart from paragraph b.), closely follows claim 1. Applicant believes that the wording of paragraph b.) in claim 24 clearly shows the relationship of the elements of the claims.

On page 3, the Office rejected claim 9 as indefinite because it is uncertain what features illustrated in Figure 1 are included and/or are excluded from the invention.

Applicant has amended claim 9 to clarify what those elements are.

On pages 3 and 4, the Office rejected claims 1, 3 to 9 under 35 U.S.C. §112, second paragraph, as failing to set forth the subject matter which applicants regard as their invention.

In particular, the view is expressed that, while the claims are drawn to the incorporation of dextran, the intended subject matter was drawn to the incorporation of glucose or dextrose, but not dextran.

In response, applicant has amended the specification to refer to dextrose rather than dextran.

In In re Oda, the court held that an amendment to correct an obvious error does not constitute new matter where one skilled in the art would recognize the existence of error in the specification as well as the appropriate correction. In re Oda, 443 F.2d 1200, 170 USPQ 260 (CCPA 1971).

Applicant submits that the person skilled in the art would have readily recognized that the reference to “dextran” as part of the aqueous solution containing citric acid and Na citrate was an error. Applicant submits that the person skilled in the art would also understand that reference was made to ACD, which contains dextrose rather than

dextran and therefore such a person would recognize the appropriate correction. ACD is a well known anticoagulant, which is widely used (Haema 2003; 6(3): 406-107, attached). For example, U.S. Patent 5,676,849 to Sammons as cited by the Office discloses that ACD is added to the blood that was collected (column 6, line 58). Also, the Office on, for example, page 4 and 9 of the Office Action made the appropriate substitution.

***Rejection under 35 U.S.C. §102(a)***

On pages 5 to 7, the Office rejected claims 1 and 3 to 8 as anticipated by Sitar et al., Cytometry, April 1, 1999, Vol. 35, No. 4, pages 337-345.

In particular, the view was expressed that this reference discloses substantially the same, if not the identical, method for isolating or preparing nucleated red blood cells.

Applicant respectfully disagrees. One, though not the only, important difference is that the disclosed method uses cord blood rather than peripheral maternal blood as required by all pending claims.

However, Gianmaria Sitar also developed the method described in this paper. Thus, to facilitate prosecution of this case, a declaration by the Gianmaria Sitar establishing that the paper is describing his own work is enclosed. The declaration is a specific affidavit (MPEP §2132.01). As the Office will note the reference to 37 CFR §1.131 in this declaration was made in error, which, however, does not affect its content and effect.

***Rejections under 35 U.S.C. §103(a)***

Claims 1, 3-5, 7 and 8 were rejected under 35 U.S.C. §103(a) as being unpatentable over U.S. Patent No. 5,641,628 to Bianchi (hereinafter "Bianchi") in view of U.S. Patent No. 5,676,849 to Sammons et al. (hereinafter "Sammons"), U.S. Patent No. 5,432,054 to Saunders et al. (hereinafter "Saunders") and Guyton's Textbook of Medical Physiology.

The view was expressed that Bianchi teaches a method for isolating nucleated fetal cells from maternal peripheral blood intended for prenatal genetic investigation (example 10), wherein the method encompasses:

- isolation of fetal nucleated red blood cells by density gradient centrifugation of maternal blood which were modified by addition of citrate dextrose aqueous solution (col. 22, line 42),
- transferring the mixture to a cell separation device,
- adding a high density liquid with Ficoll,
- isolating mononuclear cells including NRBCs by subjecting the separating device to centrifugal force,
- washing and resuspending the isolated cells, and
- identifying fetal NRBC (with antibodies to precursors of hematopoietic cells and by PCR techniques with Y chromosome primers).

On the top of page 9, the Office acknowledged that Bianchi is silent with regard to the pH value.

However, the view was expressed that Sammons ('849) makes clear that ACD contains citric acid. Thus, it was argued that the solution in the method of U.S. Patent No. 5,641,628 is reasonably expected to provide for pH lower than neutral.

Applicant submits a declaration (FN. 1) supporting that

- a. Bianchi can be expected to add tissue culture medium to his blood sample to dilute the blood cells in order to preserve them for use in the subsequent procedures described in the patent.
- b. ACD is an anticoagulant that is generally added to blood in percentiles of 15%;
- c. a standard liquid tissue culture medium, including RPMI 1640 medium, that is disclosed in Bianchi, contains buffers such as bicarbonate and/or HEPES;
- d. due to the presence of these buffers the pH of a mixture containing peripheral

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<sup>1</sup> The reference cited on page 1 of the attached declaration was submitted in an IDS on September 17, 2003; page 4 of the declaration was substituted with the consent of G. Sitar to correct minor informalities.

maternal blood, a typical tissue culture medium and the anticoagulant ACD can be expected to be around 7.0 to 7.4; and that

- e. even if Bianchi would not add tissue culture medium to the maternal blood, the addition of 15% ACD would, according to recent studies in the inventor's laboratory, still yield a pH above the claimed pH of 6.4 to 6.6, namely 6.8 - 6.9.

Thus, the above supports that the addition of regular amounts of the anticoagulant ACD to blood as disclosed in Bianchi, would not lower the pH to the claimed range of 6.4 to 6.6.

The declaration also outlines why the Example on page 10 of Sammons does not disclose the claimed pH range by inherency. The declaration supports that the mixture of Sammons used in the first centrifugation step, at the very least, when used within 24hrs will not have a pH of 6.4 to 6.6 and that the mixture used in the second centrifugation step has a pH well above the claimed range in view of the presence of PBS.

Applicant notes that a characteristic is only considered inherent if this characteristic necessarily flows from the teachings of the applied prior art. MPEP §2112 citing Ex parte Levy, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990) (emphasis added).

Thus, applicant has shown that the presently claimed invention is patentably distinct from the prior art on the basis of the pH range of the tissue culture mixture as presently claimed.

However, even if the currently claimed pH range would fall into a larger pH range that Bianchi and Sammons disclosed, what applicant denies, there is no indication in Bianchi or Sammons that pH is a result effective variable. It has been held that a result effective, i.e., a variable which achieves a recognized result has to be involved, before the determination of the optimum or workable ranges of said variable might be characterized as routine experimentation and thus be considered obvious (See, e.g., MPEP §2144.05 citing In re Antonie, 559 F.2d 618, 195 USPQ 6 (CCPA 1977)).

Applicant submit that the prior art's failure to indicate that pH is a result effective

variable is a separate reason supporting the patentability of all claims.

As the Office noted on page 8, line 4 of the Office Action, Bianchi discloses in Example 10, the isolation of a mononuclear cells layer by Ficoll/Hypaque density centrifugation from a mixture containing maternal blood. As stated in the attached declaration under 37 CFR §1.132 such a mononuclear cell layer, by definition, includes NRBCs as well as many maternal mononuclear cells such as monocytes and lymphocytes.

Thus, Bianchi does not disclose isolation of NRBCs or the separation of NRBCs from a mixture containing maternal blood by centrifugation as required by the claims:

“a.) mixing maternal blood and tissue culture medium  
to form a non-physiological tissue culture mixture having the  
following characteristics:

...  
b) transferring the non-physiological tissue culture  
mixture obtained in step a) into a cell separation device

...  
c) in discontinuous density gradient, subjecting the  
separation device to centrifugal force to isolate the NRBCs  
having a lower density than the liquid introduced in step b)”  
(**claim 1**, emphasis added)

“providing peripheral maternal blood

...  
to create a non-physiological tissue culture mixture

...  
causing separation of said NRBCs from said maternal  
blood cells by subjecting said non-physiological tissue  
culture mixture to centrifugation in a discontinuous density  
gradient” (**claim 11**, emphasis added)

Bianchi states in column 22, line 44 that the "mononuclear cell layer . . . was incubated with monoclonal antibodies directed against antigens expressed on . . . hemopoietic progenitor cell." Analysis and FLOW SORTING of fluorescent cells were performed with a FACS (Fluorescent activated cell sorter). Thus, FACS is the true isolation/separation procedure, while the isolation of mononuclear cells in the preceding centrifugation step is just a preliminary step to remove red blood cells. As a result, the



present invention (compare also Sammons ('849) and Saunders ('054)) allows that NRBCs can be separated from maternal blood in a single centrifugation step (see new claims 20 and 21).

In both Sammons ('849) and Saunders' ('054) centrifugation of a mixture containing maternal blood is only designed to remove red blood cells (Sammons) or plasma (Saunders) from a nucleated fraction (see, for example, column 10, lines 36 to 62 of Sammons and column 6, lines 20 to 30 of Saunders). Both patents, describe this centrifugation of maternal blood as a preliminary step. The true separation procedure is subsequent charge flow separation for Sammons and differential hemolysis for Saunders (column 6 line 57 and on).

Thus, neither Sammons nor Saunders disclose that the centrifugation of a mixture containing maternal blood goes beyond that of Bianchi. Thus, there is no motivation in any of the cited references nor in the general knowledge in art to modify Bianchi's centrifugation step to isolate or separate NRBCs as currently claimed, nor is there any expectation of success.

Thus, applicant submits that the cited prior art's failure alone or in combination with the general knowledge in the art to discloses or suggests isolation or separation of NRBCs from a mixture containing maternal blood by centrifugation of the mixture is a further and separate reason supporting the patentability of all claims.

Applicant also notes that Bianchi is silent as to the following limitation of claim 11:

"causing the density of said NRBCs to decrease and the cell density of said maternal blood cells to increase by transferring said maternal blood into a non-physiological liquid comprising non-physiological tissue culture to create a non-physiological tissue culture mixture" (emphasis added)

In this context it is interesting to note that, in contrast to the present invention, Saunders modifies the tonicity of the density gradient medium (see, e.g, column 7, line 66 to column 8, line 6) to change the densities of different cell types.

Also on page 9, the Office acknowledged that Bianchi is silent about the liquid

density in her centrifugation step.

However, the view was expressed that Saunders, especially in table 2, teaches the use of liquid density gradient centrifugation for the separation of fetal cells from maternal blood, wherein the liquid density gradient includes 1.065g/ml, which was considered to be about 1.068 g/ml.

Saunders discloses in Table 2, density gradient medium solutions for the preparation of the colloid/gel density gradient (see Example 2 in columns 11 and 12). However, this solution is used for Saunders' second centrifugation step (see, for example column 6, lines 57 to 59 and column 7, lines 20 to 45 of Saunders). This centrifugation step does not use a mixture containing maternal blood, but red and white blood cells isolated in a previous centrifugation step. Thus, there is no motivation to use in Bianchi's centrifugation of a mixture containing maternal blood, the density gradient disclosed in example 2 of Saunders.

The Office also acknowledged that Bianchi is silent with regard to the final characteristics of the modified blood sample as recited in claims 1 and 7.

However, the view was expressed that the claimed amount of  $\text{Cl}^-$ ,  $\text{Ca}^{++}$ , lactate and the claimed osmolarity are disclosed by Guyton, page 277. With regard to the osmolarity, the view was further expressed that the osmolarity of the ultimate solution disclosed by Bianchi should be expected to be higher than the disclosed osmolarity due to the addition of the aqueous citrate-dextrose solution.

With regard to the claimed amounts of  $\text{Na}^+$  and  $\text{K}^+$ , the Office acknowledges that the claimed amounts are the same or slightly higher than in normal blood as disclosed by Guyton. However, the view was expressed that the citrate aqueous solution of Bianchi is likely to provide for additional sodium or potassium, especially, when combined with the fact that the blood sample is stored overnight with the culture medium RPMI (col. 13, line 42).

Claim 1 discloses specific ranges of the different ingredients of the non-physiological tissue culture mixture, for example an osmolarity of 300 to 330 mOsm and a  $\text{Na}^+$  concentration of 150 to 170 mmol/l. Thus, even if the Office's above assertions

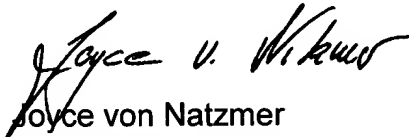
were correct, which applicant neither admits or denies, there is no indication in Bianchi or Guyton that any of the recited variables might be a result effective variable. However, it has been held that a result effective, i.e., a variable which achieves a recognized result has to be involved, before the determination of the optimum or workable ranges of said variable might be characterized as routine experimentation and thus be considered obvious (See, e.g., MPEP §2144.05 citing In re Antonie, 559 F.2d 618, 195 USPQ 6 (CCPA 1977)).

Thus, applicant submits that the prior art's failure to indicate that indicated elements are result effective variables is a separate reason supporting the patentability of claim 1.

Reconsideration of the application is respectfully requested.

The Examiner is invited to call applicant's representative at the number listed below to further the completion of the prosecution this case.

Respectfully submitted,



Joyce von Natzmer  
Attorney for Applicant  
Registration No. 48,120  
(301) 657-1282

**June 30, 2004**

4615 North Park Avenue  
Suite 919  
Chevy Chase, MD 20815

## A note in the acid citrate dextrose tube in the present usage

Viroj Wiwanitkit

Department of Laboratory Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

**Abstract.** Acid citrate dextrose (ACD) is an important blood anticoagulant and preservative. This anticoagulant is mainly used for the blood bank studies, HLA phenotyping, flow cytometry testing, tissue typing, DNA testing. The author reviews the literature on using the ACD vacuum tube in laboratory medicine. ACD is a good blood anticoagulant with few effects on analyses.

**Key words:** acide citrate dextrose • ACD • anticoagulant

✉ **Correspondence:** Viroj Wiwanitkit, MD, Department of Laboratory Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand, e-mail: Viroj.w@chula.ac.th

### INTRODUCTION

Acid citrate dextrose (ACD) is an important blood anticoagulant. At present, there are two widely used forms of acid citrate dextrose: namely solution A and solution B. Solution A comprises of 22.0 g/l trisodium citrate, 8.0 g/l citric acid, 24.5 g/l dextrose. Solution B comprises of 13.2 g/l trisodium citrate, 4.8 g/l citric acid, 14.7 g/l dextrose. This anticoagulant is used for the blood bank studies, HLA phenotyping, flow cytometry testing, tissue typing, DNA and paternity testing<sup>1</sup>. It also used as blood preservatives. For the purpose of anticoagulant for further laboratory studies, either solution A or solution B is acceptable. However, for the purpose of blood preservative, solution A is preferred. The action of this additive as anticoagulant is similar to the citrate, resulting in preservation of clotting factor while the dextrose and citric acid make ACD a good red blood cell preservative. Since it contains citrates, therefore, citric acid poisoning (hypocalcaemia) on blood preserved by ACD is mentioned. Symptoms attributable to decrease in calcium ions (numbness in fingers, nausea and, etc.) may occur after mas-

sive administration of ACD preserved blood<sup>1</sup>. In addition, ACD is not good medium for rheology studies, as it cause deformability of stored erythrocytes, resulting in bad filterability<sup>2</sup>.

In this article, the author reviews on the usage of it as anticoagulant. At present, the ACD is another application in the evacuated blood collection system, the widely used blood collection technique. Concerning the order of drawn the ACD tube should be set as the last tube (Table 1). Both solution A (8 ml draw) and solution B (3 ml draw) ACD vacuum tube is available but the solution A tube is larger.

### LITERATURE ON USING ACD TUBE IN LABORATORY MEDICINE

#### *ACD Anticoagulant Effect on Clotting Factor and Platelet*

The ACD is claimed to be a good blood anticoagulant, which can preserve the clotting factors. Lombarts and de Kieviet<sup>3</sup> reported that ACD vacuum tube can prevent unrecognized anticoagulant - induced

**Table 1.** Order of drawn.

Order	Tube	Stopper color
1.	Trace metal free tube with EDTA	Navy blue
2.	Sterile tube without additive	Pink
3.	Citrate tubes for coagulation tests	Light blue
4.	Gel (SST) tube with clot activator	Tiger
5.	Sodium-heparin tube without gel	Green
6.	Lithium-heparin tube with gel	Lime green
7.	EDTA tube	Purple
8.	Oxalate/fluoride tube	Gray
9.	Acid-Citrate Dextrose tube	Yellow

platelet aggregation, leading to pseudothrombocytopenia and concomitant pseudoleukocytosis, which EDTA and heparin generally induce. Pignatelli et al studied on the storage on in vitro platelet responses comparing to sodium citrate anticoagulant<sup>4</sup>. They concluded that the use of ACD Formula A as anticoagulant is capable of maintaining a normal platelet responsiveness up to 6-8 hours, thus permitting the investigation of platelet function for periods of time over those commonly recommended. Pignatelli et al also performed another study to evaluate whether the use of ACD Formula A may affect in vitro platelet function. They found that the ACD treated platelets showed a higher reactivity to the agonists as demonstrated by a significant increase of the maximum percentages of aggregation induced by ADP, epinephrine, and collagen, as well as a significant decrease of secondary aggregation thresholds to ADP and epinephrine<sup>5</sup>. From this study, it may be speculated that ACD Formula A is capable of better maintaining the intraplatelet signal transduction mechanisms during PRP preparation, thus improving the overall responsiveness of platelets<sup>5</sup>.

#### *ACD Anticoagulant Effect on Virology Test*

ACD has been shown to display no significant difference of virology test comparing to the standard EDTA anticoagulant. Fiscus et al studied blood samples collected in acid-citrate-dextrose and EDTA for human immunodeficiency virus (HIV) infectivity on the day of collection or after 1 day of storage at room temperature. According to this study, no significant differences between the anticoagulants were ob-

served. Culture positivity was significantly associated with HIV RNA viral loads for both anticoagulants<sup>6</sup>. Similar study from Landry et al on cytomegalovirus (CMV) virology test was performed. In this study, duplicate blood samples collected in EDTA and ACD were compared by CMV pp65 antigenaemia and CMV infectivity on the day of sample collection and after 1 and 2 days of storage at 4°C. Interestingly, no significant difference was detected between EDTA and ACD<sup>7</sup>.

#### *ACD Anticoagulant Effect on Leukocyte*

ACD anticoagulant is believed to be a good anticoagulant for leukocyte preservation. Comparison of heparin and ACD as anticoagulants revealed that ss-glucuronidase and hexosaminidase activities in plasma reached levels near the lower normal limits when ACD was used<sup>8</sup>.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Giammaria SITAR

Examiner: Vera

Afremova

Serial No.: 09/937,137

Art Unit: 1651

Filed: September 21, 2001

Atty.

Dkt.: 3026-101 (New)

For: A METHOD FOR THE SEPARATION OF FETAL CELLS FROM THE  
MATERNAL PERIPHERAL BLOOD

~~DECLARATION~~ UNDER 37 CFR §1.131

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

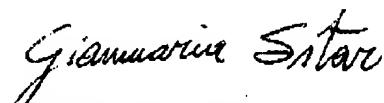
I, Giammaria Sitar, am the inventor of the above  
referenced application.

In the Office Action of December 30, 2003, the Office  
rejected claims 1 and 3 to 8 under 35 U.S.C. §102(a) as  
being anticipated by Sitar et al., Cytometry, April 1,  
1999, Vol. 35, No. 4, pages 337-45.

I believe to have solely developed the cell separation  
method disclosed in this paper under the headings "Blood  
Sample Collection and Nucleated Cell Separation" and  
"Separation of mononuclear cells by isopyknic gradient  
centrifugation" on pages 338 to 339 of this paper.

I, Gianmaria Sitar, declare that all statements made herein that are based on my own knowledge are true and all statements made on information and belief are believed to be true. I acknowledge that willful false statements are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

  
Gianmaria Sitar

Date: 6/29/04

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Giammaria SITAR

Examiner: Vera

Afremova

Serial No.: 09/937,137

Art Unit: 1651

Filed: September 21, 2001

Atty.

Dkt.: 3026-101 (New)

For: A METHOD FOR THE SEPARATION OF FETAL CELLS FROM THE  
MATERNAL PERIPHERAL BLOOD

## DECLARATION UNDER 37 CFR §1.132

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

I, Giammaria Sitar, am a professor of medicine at the University of Pavia, Italy. I have been working on the subject of separating different cell types from blood since 1974 and have co-authored more than ten papers that deal with this subject.

The presently claimed invention requires that the claimed tissue culture mixture containing peripheral maternal blood has a pH of 6.4 to 6.6. This pH range will allow for the separation of fetal nucleated red blood cells (NRBCs) from maternal blood cells such as monocytes and lymphocytes which have cell densities very similar to NRBCs (Haematologica 1997,82: 5-10: "Characterization of the biophysical properties of human erythroblasts as a preliminary step to the isolation of fetal erythroblasts from maternal peripheral blood for non-invasive prenatal genetic investigation"). The claimed pH range ensures that the NRBCs become lighter and maternal blood cells with similar density distribution profiles





become heavier so that they can be readily separated during subsequent discontinuous density gradient centrifugation. This fact allows that NRBCs can be separated in a single centrifugation step. On information and belief, in all other procedures that I am aware of, the initial step of discontinuous density gradient centrifugation of maternal blood does not provide an enrichment of NRBCs but is performed to remove the bulk of red blood cells from maternal blood (there are about 125 BILLIONS red blood cells in 25 ml maternal blood) to avoid an overload of the system in the procedure that is subsequently used.

The below supports that the maternal blood cells containing mixtures that are subjected to centrifugation disclosed in U.S. Patents 5,641,628 and Patent 5,676,849 will not attain a pH within the presently claimed pH range. This supports that the methods disclosed in these patents will not allow for the separation of NRBCs from maternal blood cells or their isolation according to the presently claimed invention.

U.S. Patent No. 5,641,628 to Bianchi et al. discloses in example 10, starting in column 22, a method for detecting fetal stem cells in maternal circulation. On information and belief, the initial step of discontinuous density gradient centrifugation (column 22 line 44-45) is limited to removing red blood cells, while the true isolation technology is described to be the flow sorting of fluorescent cells (column, 22 line 50). In particular, the patent describes that mononuclear cells, isolated by discontinuous density gradient centrifugation, were incubated with monoclonal antibodies directed antigens expressed on precursors progenitor cells and flow sorted by FACS (Fluorescent activated cell sorter). Based on information I obtained during my work in the field of cells separation, I believe it to be technically impossible to use FACS if red blood cells are not previously removed.

Example 10 discloses collecting venous blood (20ml) in citrate dextrose (ACD-A) and subsequent centrifugation. I believe that Bianchi adds tissue

culture medium such as standard liquid form of RPMI 1640 before adding ACD-A since this is, according to information I obtained during my work in the field of cell separation, standard procedure. It is my belief that it is clear that Bianchi uses citrate dextrose as anticoagulant. On information and belief, the normal percentile of anticoagulants generally added to blood is around 15%. While ACD has a pH of 4.4, a mixture of 3.75 ml of ACD (15%) with 25ml peripheral blood, which has a pH of around 7.39, results according to information obtained during recent studies performed in my laboratory, in a pH of 6.8 - 6.9.

In column 13, line 42, Bianchi discloses the use of RPMI 1640 medium containing lithium heparin (10IU/ml). The standard liquid form of RPMI 1640, which is, on information and belief, generally used as tissue culture medium, contains bicarbonate and/or HEPES as buffers. On information and belief, only specialized formulations such as the 10x concentrated solution, the powered version or the modified version will not contain either sodium bicarbonate and/or HEPES as a buffer (see attached printout from Sigma-Adrich website). These buffers will prevent any substantial reduction in the pH of blood subsequent to, for example, the addition of ACD as an anticoagulant. Thus, on information and belief, if standard tissue culture medium and ACD is added to peripheral maternal blood, the pH will lie even higher than 6.8 - 6.9, probably in the range of pH 7.0 - 7.4.

I believe that column 13 of Bianchi confirms that mononuclear cell separation is indeed only an initial step since she writes about the discussed prior art (line 54-55) "An advantage to this particular technique is that an initial step which results in mononuclear cell isolation is not added".

Bianchi discloses the isolation of a mononuclear cells layer after centrifugation. On information and belief, such a mononuclear cell layer will not only contain fetal NRBCs, but also many maternal mononuclear cells such as monocytes and lymphocytes. This further supports that Bianchi's method does

presently claimed. Indeed discontinuous density gradient centrifugation is only a preliminary step. On information and belief, Bianchi's true separation is not based on density gradient centrifugation but on FACS (Fluorescent activated cell sorter).

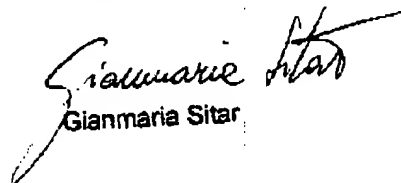
U.S. Patent 5,676,849 to Sammons et al. discloses a method for the enrichment of fetal cell population from maternal whole blood samples. The Example spanning columns 10 and 11 includes two centrifugation steps. The first one is described in column 10, lines 36 to 62. Here 40ml blood is collected in an anticoagulant similar to those described earlier in column 6, lines 55 to 60, namely CPD (2.55g d-glucose, 2.63g sodium citrate, 0.327g citric acid and 0.222 monobasic sodium phosphate in 100 ml distilled water). The so collected blood is layered on HISTOPAQUE-1119 and centrifuged. Sammons explicitly states that CPD is used as an anticoagulant. Again the goal of this first centrifugation step is to remove red blood cells from a maternal blood sample. While I have not personally worked with CPD, I believe from my work with ACD, that the solution prepared in this first step of the example in column 10, lines 36 to 62, does not have the pH of the tissue culture mixture currently claimed. I believe that it is almost certain that such a mixture will not necessarily have a pH in the claimed range. My belief is based, among others, on my observation that blood cells in the claimed tissue culture mixture having pH of 6.4 to 6.6 generally begin to die after 24hrs. In column 6, lines 51 to 65, Sammons discloses that the blood collected in one of the anticoagulants listed, including CPDA, can be stored for 4 day at 4°C, which, based information obtained during my studies, implies that if this type of collected blood is used within 24hrs as Sammons suggests in this paragraph, this collected blood has a pH higher than 6.6, most likely higher than 7.0. Also, blood, even if not combined with a standard tissue culture medium, has a natural buffering capacity, which, on information and belief, would prevent a drop of the

pH of blood for some time even if acids are added, unless a specific effort is made such by the addition of non-buffered culture medium.

In the second centrifugation step described in column 10, line 63 to column 11, line 1, PBS (phosphate buffered solution) is added to the nuclear fraction obtained from the above described centrifugation step and the mix is then centrifuged. PBS has generally a pH of 7.2 - 7.4. The mix of the nuclear fraction and PBS thus has a pH well above the claimed range. On information and belief, in Sammons, the NRBCs only get truly separated during the charge flow separation (CFS) step disclosed in column 11, lines 2 to 12.

I, Gianmaria Sitar, declare that all statements made herein that are based on my own knowledge are true and all statements made on information and belief are believed to be true. I acknowledge that willful false statements are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

  
Gianmaria Sitar

June 30, 2004



## Life Science

- + Antibody Explorer
- + Automation
- Books
- + Cancer Research
- Cell Culture
  - Product Lines

- Antibody Expression
- Classic Media & Salts
- ECACC Cell Lines
- Embryo Culture
- Equipment
- Gene Therapy
- Primary Human Culture
- Protein Expression/CHO
- Reagents & Supplements
- Sera
- Serum Free Media
- Stem Cells
- SyntheChol NS0
- TrypZean Solution
- Vaccine Production

- + Product Highlights
- + Key Resources
- + Cell Signaling
- Custom Synthesis
- Drug Discovery
- + Molecular Biology
- + Plant Biotechnology
- + Proteomics and Protein Expr.
- + Life Science Quarterly

## CLASSIC MEDIA AND SALTS

## Cell Culture

Basal Salt Mixtures :: Dulbecco's Media :: Ham's Nutrient Mixtures :: MCDB  
MEM Media :: Medium 199 :: MegaCell™ Media :: Other Classic Media :: RPMI

## RPMI Media

RPMI-1640 was developed by Moore et. al. at Roswell Park Memorial Institute, hence the name RPMI. The formulation is based on the RPMI-1630 series of media utilizing a bicarbonate system and alterations in the amounts of amino acids and vitamins. RPMI-1640 medium is used for the culture of human normal and neoplastic leukocytes. RPMI-1640, when properly supplemented, has demonstrated wide applicability for supporting growth of many type of cells, including fresh human lymphocytes in the 72 hour phytohemagglutinin (PHA) stimulated

Product #	Product Name	Description	Application
R 0883	RPMI-1640 Medium	<ul style="list-style-type: none"> <li>• Liquid</li> <li>• With sodium bicarbonate</li> <li>• Without L-glutamine; supplement with 0.3 gm/L L-glutamine</li> <li>• Sterile-filtered</li> <li>• Endotoxin tested</li> <li>• Cell culture tested</li> </ul>	Same as R 8758, except glutamine must be added by investigator.
R 8758	RPMI-1640 Medium	<ul style="list-style-type: none"> <li>• Liquid</li> <li>• With sodium bicarbonate and L-glutamine</li> <li>• Sterile-filtered</li> <li>• Endotoxin tested</li> <li>• Cell culture tested</li> </ul>	Complete liquid version of RPMI-1640 formulation as described by Moore et. al. in 1967.
R 1145	RPMI-1640 Medium (10x)	<ul style="list-style-type: none"> <li>• Liquid</li> <li>• Without L-glutamine, folic acid and sodium bicarbonate</li> <li>• Supplement with 0.3 gm/L L-glutamine, 0.001gm/L folic acid and 2.0 gm/L sodium bicarbonate at 1x</li> <li>• pH 1.8-2.2 at 10x</li> <li>• Sterile-filtered</li> <li>• Endotoxin tested</li> <li>• Cell culture tested</li> </ul>	This is the concentrated version of R 8758. The L-glutamine and sodium bicarbonate are added to the medium after dilution to 1x and properly mixed.
R 6504	RPMI-1640 Medium	<ul style="list-style-type: none"> <li>• Powder</li> <li>• With L-glutamine</li> <li>• Without sodium bicarbonate; supplement with 2.0 gm/L sodium bicarbonate.</li> <li>• Formulated at 10.4 grams of powder per liter of medium.</li> <li>• Cell culture tested</li> </ul>	Related to R 5382, RPMI Hybri-Max.
R 7509	RPMI-1640 Medium Modified	<ul style="list-style-type: none"> <li>• Liquid</li> <li>• With sodium bicarbonate</li> </ul>	Phenol red has been shown to be inhibitory to cell growth.

		<ul style="list-style-type: none"> <li>Without phenol red and L-glutamine; supplement with 0.3 gm/L L-glutamine.</li> <li>Sterile-filtered</li> <li>Endotoxin tested</li> <li>Cell culture tested</li> </ul>	<p>with the growth of some densities.</p> <p>Use this medium when stem cells or when grow densities.</p> <p>Also recommended for diagnostics use.</p>
R 5886	RPMI-1640 Medium HEPES Modification	<ul style="list-style-type: none"> <li>Liquid</li> <li>With 25 mM HEPES and sodium bicarbonate</li> <li>Without L-glutamine; supplement with 0.3 gm/L L-glutamine.</li> <li>Sterile-filtered</li> <li>Endotoxin tested</li> <li>Cell culture tested</li> </ul>	<p>Same as R 8758, except buffer has been added.</p> <p>HEPES increases the buffering range.</p> <p>Recommended for cells better at a pH above 7.2</p>
R 4130	RPMI-1640 Medium HEPES Modification	<ul style="list-style-type: none"> <li>Powder</li> <li>With L-glutamine and 25 mM HEPES</li> <li>Without sodium bicarbonate; supplement with 2.0 gm/L sodium bicarbonate.</li> <li>Formulated at 16.4 grams of powder per liter of medium.</li> <li>Cell culture tested</li> </ul>	<p>Same as R 8758, except buffer has been added.</p> <p>HEPES increases the buffering range.</p> <p>Recommended for cells better at a pH above 7.2</p>
R 7638	RPMI-1640 Medium Dutch Modification	<ul style="list-style-type: none"> <li>Liquid</li> <li>With 1 gm/L sodium bicarbonate and 20 mM HEPES</li> <li>Without L-glutamine; supplement with 0.3 gm/L L-glutamine.</li> <li>Sterile-filtered</li> <li>Endotoxin tested</li> <li>Cell culture tested</li> </ul>	<p>Same as R 8758, except concentration of sodium has been cut in half and has been added.</p> <p>HEPES increases the buffering range.</p> <p>Recommended for cells better at a pH above 7.2</p>
R 7388	RPMI-1640 Medium Modified	<ul style="list-style-type: none"> <li>Liquid</li> <li>With 20 mM HEPES and L-glutamine</li> <li>Without sodium bicarbonate; supplement with 2.0 gm/L sodium bicarbonate.</li> <li>Sterile-filtered</li> <li>Endotoxin tested</li> <li>Cell culture tested</li> </ul>	<p>Same as R 8758, except buffer has been added.</p> <p>HEPES increases the buffering range.</p> <p>Recommended for cells better at a pH above 7.2</p>
R 8755	RPMI-1640 Medium Modified	<ul style="list-style-type: none"> <li>Powder</li> <li>With L-glutamine</li> <li>Without phenol red and sodium bicarbonate; supplement with 2.0 gm/L sodium bicarbonate.</li> <li>Formulated at 10.4 grams of powder per liter of medium.</li> <li>Cell culture tested</li> </ul>	<p>Phenol red has been shown with the growth of some densities.</p> <p>Use this medium when stem cells or when grow densities.</p> <p>Also recommended for diagnostics use.</p>
R 7755	RPMI-1640 Medium Auto-Mod?	<ul style="list-style-type: none"> <li>Powder</li> </ul>	Modified for autoclaving

		<ul style="list-style-type: none"><li>• Without L-glutamine and sodium bicarbonate; supplement with 0.3 gm/L of L-glutamine and 2.0 gm/L sodium bicarbonate.</li><li>• Formulated at 10.3 grams of powder per liter of medium.</li><li>• Cell culture tested</li></ul>	
R 1383	RPMI-1640 Medium	<ul style="list-style-type: none"><li>• Powder</li><li>• With L-glutamine</li><li>• Without glucose and sodium bicarbonate; supplement with 2.0 gm/L sodium bicarbonate.</li><li>• Formulated at 8.4 grams of powder per liter of medium.</li><li>• Cell culture tested</li></ul>	Without glucose, allows add own energy source.
R 7513	RPMI-1640 Medium Modified	<ul style="list-style-type: none"><li>• Liquid</li><li>• Without sodium bicarbonate</li><li>• Without L-methionine, L-cystine, and L-glutamine</li><li>• Supplement with 0.652 gm/L L-cystine-2HCL, 0.015 gm/L L-methionine, and 0.3 gm/L of L-glutamine</li><li>• Sterile-filtered</li><li>• Endotoxin tested</li><li>• Cell culture tested</li></ul>	Formulated without sulf amino acids.  Recommended for meta using radio-labeled sulf amino acids.

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